Journal of Experimental Botany, Vol. 56, No. 418, pp. 2095–2105, August 2005
doi:10.1093/jxb/eri208 Advance Access publication 13 June, 2005

Journal of Experimental Botany

RESEARCH PAPER

Analysis of indole-3-butyric acid-induced adventitious root formation on *Arabidopsis* stem segments

EXHIBIT A

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Received 24 November 2004; Accepted 27 April 2005

Abstract

Root induction by auxins is still not well understood at the molecular level. In this study a system has been devised which distinguishes between the two active auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). IBA, but not IAA, efficiently induced adventitious rooting in *Arabidopsis* stem segments at a concentration of 10 μM. In wild-type plants, roots formed exclusively out of calli at the basal end of the segments. Root formation was inhibited by 10 μM 3,4,5-triiodobenzoic acid (TIBA), an inhibitor of polar auxin transport. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trp1*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels. By contrast, two mutants of *Arabidopsis* with measurably higher levels of IAA (*trp2*, *amt1*) show root induction characteristics very similar to the wild type. Using differential display, transcripts specific to the rooting process were identified by devising a protocol that distinguished between callus production only and callus production followed by root initiation. One fragment was identical to the sequence of a putative regulatory subunit B of protein phosphatase 2A. It is suggested that adventitious rooting in *Arabidopsis* stem segments is due to an interaction between endogenous IAA and exogenous IBA. In stem explants, residual endogenous IAA is transported to the basal end of each segment, thereby inducing root formation. In stem segments in which the polar auxin transport is inhibited by TIBA, root formation does not occur.

Key words: Adventitious root formation, *Arabidopsis*, auxin, auxin-inducible proteins, differential display, indole-3-butyric acid, protein phosphatase 2A, TIBA.

Introduction

Root development in *Arabidopsis thaliana* has been the subject of many studies employing mutant screens during the last few years (for a review see Casson and Lindsey, 2003). While development of the primary root from the embryonic stage has received a lot of attention and the processes involved are beginning to unravel, the formation of lateral and adventitious roots is less well understood. Lateral and adventitious roots are formed post-embryonically. While lateral roots typically form from the root pericycle, adventitious roots form naturally from stem tissue. Adventitious roots are less predictable in their cellular site of origin than lateral roots. They may form from the cambium or, in the case of detached stem cuttings, from calli. Therefore it appears that adventitious roots can be formed by two different pathways: (i) direct organogenesis from established cell types or (ii) from callus tissue following mechanical damage (Casson and Lindsey, 2003, and references therein).

Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root. (Davies *et al.*, 1994; Kovar and Kuchenbuch, 1994). The auxin indole-3-acetic acid (IAA) was the first plant hormone to be used to stimulate rooting of cuttings (Cooper, 1935). At that time it was discovered

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that a second, 'synthetic' auxin indole-3-butyric acid (IBA) also promoted rooting and was even more effective than IAA (Zimmerman and Wilcoxon, 1935). IBA is now used commercially worldwide to root many plant species (Hartmann *et al.*, 1990). Since its introduction more than 50 years ago, IBA has been the subject of many experiments, mostly involving trial and error studies to achieve optimum rooting conditions for the plant species in question. Application of IBA to cuttings of many plant species results in the induction of adventitious roots, in many cases more efficiently than IAA (Epstein and Ludwig-Müller, 1993). For example, in *Vigna radiata* the induction of adventitious roots was observed after IBA, but not IAA application (Riov and Yang, 1989). The greater ability of IBA to promote adventitious root formation compared with IAA has been attributed to the higher stability of IBA versus IAA both in solution and in plant tissue (Nordström *et al.*, 1991). The effective concentration of IBA in these kinds of studies was also dependent on the pH of the medium. It was shown that, at lower pH values, lower IBA concentrations in the medium were sufficient to induce rooting of apple cuttings (Harbage and Stimart, 1996).

Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism (Alvarez *et al.*, 1989; Blazkova *et al.*, 1997; Epstein and Ludwig-Müller, 1993). It was shown, for example, that a difficult-to-root cultivar of *Prunus avium* conjugated IBA more rapidly than an easy-to-root cultivar (Epstein *et al.*, 1993). Only in the easy-to-root cultivar was the appearance of free IBA observed after several days and the authors concluded that the difficult-to-root cultivar was not able to hydrolyse IBA conjugates during the appropriate time points of adventitious root development. Interestingly, it was possible to induce rooting of the difficult-to-root cultivar after application of an inhibitor of conjugation (Epstein *et al.*, 1993). It has been shown that IBAsp is even more active than free IBA in the promotion of adventitious roots in mung bean, possibly due to its higher stability during the rooting process (Wiesman *et al.*, 1989). However, other differences such as uptake and transport can also account for the differences in rooting behaviour (Epstein and Ludwig-Müller, 1993).

The physiological events leading to root initiation may be revealed by using targeted or untargeted molecular approaches to identify genes that may be involved in adventitious rooting. IBA has been identified as a natural substance in *Arabidopsis thaliana* (Ludwig-Müller *et al.*, 1993) and there are indications that at least part of the action of IBA is not through IAA in this species (Poupart and Waddell, 2000; Zolman *et al.*, 2000). Therefore a system has been devised for adventitious root formation on stems of the model plant *Arabidopsis* under sterile conditions, where roots are specifically induced after the application of IBA but not of IAA. The results have shown that (i) IBA is one important factor in *Arabidopsis* to induce adventitious roots, (ii) the timing of auxin application is important to distinguish between callus and root formation, and (iii) this system is suitable for identifying genes involved in adventitious root formation. Finally, the effect of an auxin transport inhibitor, TIBA, on IBA-induced adventitious root formation has been investigated and IAA-deficient mutants were used to analyse the interplay between IAA and IBA during adventitious rooting.

Materials and methods

Plant material

Arabidopsis plants were grown aseptically on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) in Magenta® boxes at 24 °C under constant illumination with cool-white fluorescent lights, approximately 40 μmol m^{-2}. The seeds were surface-sterilized with 5% (v/v) commercial bleach (Clorox; a 5% solution of sodium hypoclorite) for 20 min, washed thoroughly, planted on 1% agar, and vernalized for 24 h at 4 °C. Inflorescenses from 4–8-week-old-plants were used because, during this period, the age of the stems did not influence callus/root formation, although on stem segments of older plants no root formation could be observed; data not shown). The inflorescences were cut into 0.5 cm node-free segments and incubated in the dark or under constant illumination in Petri dishes containing full-strength MS agar containing the appropriate concentrations of IAA or IBA with or without different concentrations of 3,4,5-triiodobenzoic acid (TIBA). In the light, the plates were covered with yellow plastic to prevent photo-oxidation of auxins (Campanella *et al.*, 1996). Starting at 5 d, plates were examined daily and the proportion of segments showing callus or root formation was scored.

For the differential display experiments, segment length was reduced to 3 mm to increase the number of ends per fresh weight. For the subsequent treatments, segments were transferred under sterile conditions to fresh Petri dishes containing either plain MS agar or MS agar with the appropriate hormone supplement.

For histology, stem segments were fixed for at least 24 h in FAA (5% formaldehyde, 5% acetic acid, 50% ethanol), then dehydrated through a series of ethanol steps (70%, 80%, 95%) before infiltration with JB-4 resin (Polysciences, Inc., Niles, IL). Sections of 2–4 μm were stained with toluidine blue.

Evaluation of the rooting process

On each Petri dish for the different treatments 10–12 *Arabidopsis* stem segments were placed. Each experimental condition consisted of at least two Petri dishes. All experiments were performed at least three times, resulting in a minimum of 60 segments which were scored per treatment. Mean values of the three independent experiments are given. After the different treatments the *Arabidopsis* stem segments were inspected for callus or root formation and the number of segments exhibiting the respective organs counted.

RNA extraction and differential display

Isolation of total RNA was performed using TRIzol reagent (Gibco BRL, now marketed by Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using 300 mg fresh weight of treated and control segments. Reverse transcription followed by PCR using anchored VNT$_1$, 3'-primers and 10-mer OPA 5'-primers (both Operon Technologies) was performed essentially as described by Liang and Pardee (1992). ^{35}S-Radiolabelled amplification products were resolved on 6% acrylamide sequencing gels and detected by autoradiography. The experiment was repeated to show reproducibility of fragment induction. Fragments induced only under condition C

were excised, re-amplified with the same primer combination, the PCR products purified (QIAquick® gel extraction kit, Qiagen), ligated into pBSK vector, and sequenced from both ends at The Institute for Genomic Research.

Northern blot analysis

Total RNA was isolated as described above. The synthesis of the biotinylated (bio-dUTP, Boehringer Mannheim) cDNA probe used for northern hybridization was performed by PCR. Template was cDNA prepared from total RNA of *Arabidopsis* stems induced with IBA. For amplification of the phosphatase 2A-like protein subunit as a probe, the following primer pair was designed according to the sequence information obtained: forward 5'-GATCATGTGATA-GAAGATAAATTTAGTGCT-3'; reverse 5'-TCTTCTATCACAT-GATCTCGTCAGGGACCA-3'. PCR was performed according to standard procedures using the following programme: initial denaturation at 96 °C for 5 min, followed by 30 cycles of 96 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s. Equal sample loading (20 μg total RNA) was confirmed by hybridization with an *actin 2* (At3g18780) probe amplified with the following primers: forward 5'-GAAGAT-TAAGGTCGTTGCACCACCTG-3'; reverse 5'-ATTAACATTG-CAAAGAGTTTCAAGGT-3'. Non-radioactive northern blots were performed according to Löw and Rausch (1994), with the Northern-Light™-kit from Tropix (Serva) for detection.

Results

Indole-3-butyric acid can induce adventitious roots on Arabidopsis stem segments

Several reports deal with the better performance of IBA versus IAA during the rooting process. This was attributed to parameters such as stability, transport, or metabolism. Therefore a protocol was devised which would induce adventitious roots on *Arabidopsis* stems by one of the auxins but not the other. This study's experiments showed that several parameters influenced adventitious root induction and helped to discriminate between the actions of IAA and IBA. These were: (i) concentration of the hormone, (ii) duration of treatment, (iii) priming event, and (iv) second hormone treatment.

In a first set of experiments, 0.5 cm explants of *Arabidopsis* stems were incubated for 7 d on MS medium containing either IAA or IBA at different concentrations and the phenotype was recorded (Fig. 1A). Since the explants looked similar when they were cultivated on hormone plates for 7 d, only the explants on different IBA concentrations are shown. The induction of adventitious roots was always preceded by callus formation. Root induction was seen at 1 μM and 10 μM IBA and IAA, and at 100 μM hormone the roots looked stunted with more root hairs produced (Fig. 1A). Similarly, root induction by IAA or IBA was also possible using excised leaves (Fig. 1B). The concentration dependence was also comparable with that for stem segments.

On stem segments treated with IBA, adventitious roots clearly arose from the cambium, which first de-differentiates to form a callus (Fig. 2B). This is followed by the formation

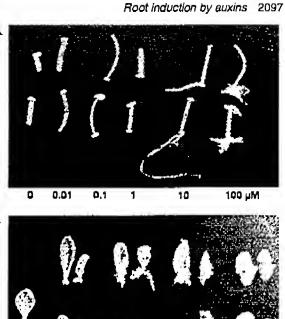

Fig. 1. Root induction on *Arabidopsis* stem (A) and leaf (B) explants after treatment with different IBA concentrations.

of roots (Fig. 2C) that subsequently elongated and by the formation of additional callus areas, which gave rise to new adventitious roots (Fig. 2D). In the controls without IBA such structures were never visible (Fig. 2A).

Timing of hormone requirement for adventitious rooting

To determine the period of IBA exposure required for adventitious root induction, the stem segments were incubated on 10 μM IAA or IBA for different time periods up to 48 h and then transferred to hormone-free MS medium for the remaining time. Callus and root formation was scored at 7 d (Fig. 3). The proportion of explants forming callus increased up to 100% after 48 h on auxin-containing medium (Fig. 3A). While callus formation was comparable on IAA- or IBA-containing MS agar, root formation was found only when IBA was in the medium. After a 6 h exposure, a response was already found, but optimum rooting was observed with a treatment of 48 h (Fig. 3B). After longer incubation periods the difference between IAA and IBA treatment became less pronounced (data not shown). The inset in Fig. 3B shows a picture of stem segments incubated for the respective time on either 10 μM IAA or 10 μM IBA.

A two-stage treatment was developed to distinguish between callus and root induction by IBA (Fig. 4). In stage I,

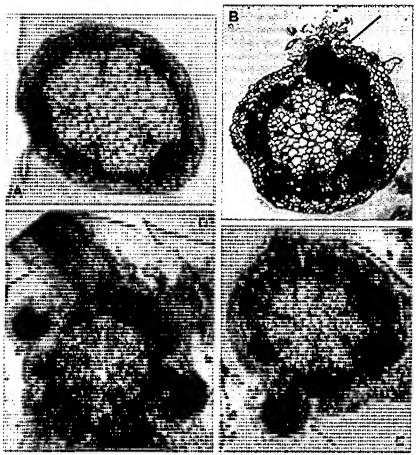

Fig. 2. Development of adventitious roots on *Arabidopsis* stems without (A) and after treatment with 30 μM IBA (B–D). Sections were taken 3 d (B), 5 d (C), and 9 d (D) after placing the segments on rooting medium. Sections of 2–4 μm were stained with toluidine blue. Adventitious roots are marked by arrows.

explants were incubated for 24 h on 10 μM IBA, a treatment that resulted in callus formation. In stage III, explants were given a second 10 μM IBA treatment of variable duration after a period of 24 h on hormone-free medium (stage II). The explants were transferred to hormone-free medium after the second IBA treatment for the remainder of the experiment (stage IV) and root formation was scored 14 d after the start of the second treatment. The second treatment resulted in the formation of adventitious roots on 60–95% of the explants, provided that it was at least 48 h long (Figs 4A, 5A). In addition, it was shown that the highest rooting efficiency was found with treatments that involved two exposures to IBA separated by a time without hormone (Fig. 5A). Increasing the incubation time of the second treatment on IBA also resulted in more segments showing adventitious root formation. Interestingly, in the experiments using only one long IBA treatment (Fig. 5B–D), more roots were formed when the treatment started with MS medium alone.

The auxin concentration was also important for the second treatment in which the explants were incubated for 48 h with different concentrations of IBA. Again with 1 μM and 10 μM IBA good induction of adventitious rooting was found with up to 95% of the segments showing roots (Fig. 5B). Callus formation without subsequent root formation was observed at concentrations <0.1 μM IBA.

Identification of transcripts expressed during adventitious rooting using differential display

The treatments of *Arabidopsis* stems described above were used to test this system for its suitability to isolate differentially expressed genes during adventitious rooting. Since the experimental procedure allowed the difference between callus formation and adventitious rooting to be distinguished, the comparison of control stems with stems treated to form callus or adventitious roots should provide transcripts which are specific for the rooting process. The

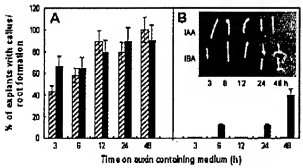

Fig. 3. Callus (A) and root (B) formation after continuous treatment with 10 μM IAA (hatched bars) or IBA (black bars) for different times on MS medium. The photograph shows the phenotype of rooted stem segments incubated for different periods on 10 μM IAA or IBA.

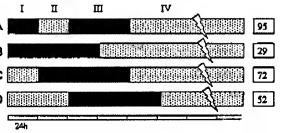

Fig. 4. Adventitious root formation is increased by a two-stage treatment and does not require continuous exposure to IBA. Different treatments with IBA are marked as follows: (dotted section) MS only, (black section) MS+10 μM IBA. The different variations tested are: (A) 24 h IBA/24 h MS/48 h IBA/MS; (B) 72 h IBA/MS; (C) 24 h MS/72 h IBA/MS; (D) 48 h MS/72 h IBA/MS. The segments were placed either on MS medium or MS supplemented with IBA after the indicated time periods (see time scale; one white bar segment represents 24 h). Percentage of root formation under the respective treatment conditions is given to the right of the bar. The stages mentioned in the text are indicated above the respective bar in Roman numerals. I: first IBA treatment; II: first period on MS; III: second IBA treatment; IV: remaining time until roots are visible on MS). The flash indicates a discontinuous time scale.

following three tissue samples were compared: (i) untreated segments; (ii) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; and (iii) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA (see Fig. 4, Regime A), which induces roots in a large fraction of the explants (Fig. 6A). For the differential display experiment, a set of arbitrary primers (OPA1-12) was used in combination with anchor primers on each of the three mRNA populations described above. Bands specific to treatment C (root induction) were obtained with OPA primers 1, 6, and 12 (data not shown). Fragments designated 01-a, 01-b, 06-a, and 12-a were excised, reamplified and further analysed. It was not possible to reamplify fragment 12-a, therefore only three differentially expressed fragments remained. In all three cases only short fragments

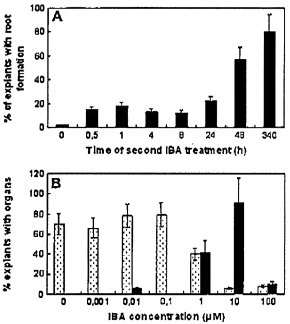

Fig. 5. Two-stage treatment for distinction between callus (dotted bars) and root (black bars) formation. (A) Dependence of root formation on the duration of the second 10 μM IBA treatment. Segments were transferred from IBA-containing medium to medium without hormone after the respective time periods. (B) Optimum concentration of IBA for the second treatment (340 h). The medium for the second treatment was supplemented with different IBA concentrations.

were amplified from the 3′-end. Therefore, all sequences are 3′-UTRs of the respective cDNAs. Since the completion of the *Arabidopsis* genome sequencing project, identification of gene sequences has been much facilitated. One 390 bp fragment (01-a) was homologous to a regulatory subunit B of protein phosphatase 2A (At3g54930). A second 340 bp fragment (01-b) was found to be derived from At1g29470 which was annotated as similar to the early-responsive dehydration stress protein, ERD3 that contains a putative methyltransferase motif. A third 300 bp fragment (06-a) was derived from At5g48545, a gene encoding an unknown protein of the histidine triad family protein with a HIT domain (http://www.tigr.org/tdb/e2k1/ath1/). Expression analysis confirmed the presence of the PP2A homologous mRNA specifically in tissues after IBA-induced adventitious root formation (Fig. 6B).

The polar auxin transport inhibitor TIBA inhibits adventitious root formation

Factors important for the effect of auxins during rooting might be (i) synthesis, (ii) metabolism, and (iii) transport. The latter was tested by using the polar auxin transport inhibitor 3,4,5-triiodobenzoic acid (TIBA) concomitantly with the IBA treatment leading to adventitious roots. Inhibition of root formation was observed when varying

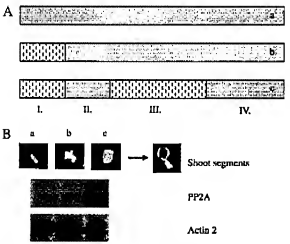

Fig. 6. Identification of transcripts specifically expressed during adventitious root formation by IBA. (A) The different treatment of segments is shown: (a) untreated segments; (b) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; (c) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA. (dashed section) Time on IBA, (grey section) MS only. (B) RNA expression analysis of one fragment (PP2A B-regulatory subunit) in the three different tissues. The small photographs (a–c) show the segments as they looked after the harvest. Root development in treatment (c) was only observed later (indicated by an arrow). The Roman numerals correspond to those in Fig. 4.

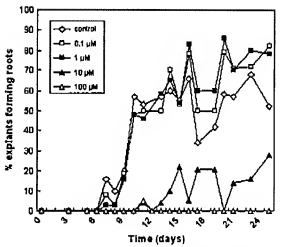

Fig. 7. Adventitious root formation at different concentrations of the IAA transport inhibitor TIBA in wild-type plants over a time period of 4 weeks. IBA was always at 10 μM. Inhibition of adventitious root formation was found at equimolar concentrations of IBA and TIBA.

concentrations of TIBA were added together with a fixed concentration of IBA (10 μM) in the medium (Fig. 7). While 0.1 μM and 1 μM TIBA had no inhibitory effect, 10 μM TIBA was already inhibitory and 100 μM TIBA completely prevented adventitious root formation. With lower TIBA concentrations there even seemed to be a small promoting effect after longer incubation times.

Arabidopsis *mutants with altered adventitious root formation*

IBA is an important factor for adventitious root formation if applied exogenously. However, endogenous auxins may also play a role in the rooting process. Therefore three mutants with altered auxin levels were investigated for their ability to form adventitious roots after IBA treatment. The mutant *amt1* (Kreps and Town, 1992) has no altered phenotype compared with the wild type when grown under normal conditions. However, if *amt1* was grown on 10 μM IBA, the roots looked more stunted with a higher number of lateral roots and, at higher concentrations, less root growth than the wild type was observed. *amt1* also showed altered levels of IAA and IBA (Ludwig-Müller *et al.*, 1993). It was therefore of interest to test whether this mutant behaved differently concerning adventitious rooting and so at the same time two other mutants with defects in the tryptophan biosynthesis pathway, *trp1* and *trp2* (Last *et al.*, 1991; Rose *et al.*, 1992) were included. Since adventitious root formation was shown to be concentration-dependent in *Arabidopsis*, several IBA concentrations were tested on wild-type and mutant stem segments. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trp1*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels, compared with wild-type Columbia (Fig. 8). The two other mutants (*amt1* and *trp2*) with measurably higher levels of IAA show root induction characteristics very similar to the wild type.

Discussion

Arabidopsis has been used for the investigation of lateral root development (Neuteboom *et al.*, 1999) because of its relatively simple organization of both primary and lateral roots (Dolan *et al.*, 1993). Lateral root formation in root cultures of *Arabidopsis* was initiated by exogenous auxin. Differential screening of a cDNA library from roots treated with 1-NAA and the inactive analogue 2-NAA led to the isolation of four cDNAs clones coding for proteins putatively active outside the cell such as subtilisin-like serine protease (Neuteboom *et al.*, 1993). *Arabidopsis* mutants exhibiting more lateral roots (*sur1*, *sur2*) were linked to an overproduction of IAA (Boerjan *et al.*, 1995; Delarue *et al.*, 1998). However, other genes regulated independently of auxin induction are also involved in lateral root development, such as the nuclear-localized protein ALF4 (DiDonato *et al.*, 2004).

Evidence for the involvement of IBA, but not IAA, in lateral root development was recently reported for lateral root induction in rice (Wang *et al.*, 2003). While IBA was

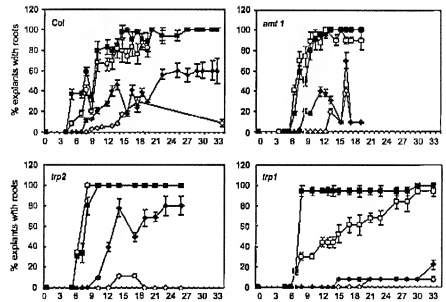

Fig. 8. Adventitious root induction at different IBA concentrations in wild type (Columbia) and three different *Arabidopsis* mutants with altered auxin content over a time period of 5 weeks: (open diamonds) 1 μM, (filled diamonds) 3 μM, (open squares) 10 μM, (filled squares) 30 μM.

able to induce lateral roots, the same response was found only at 20-fold higher concentrations of IAA (Chhun *et al.*, 2003, 2004). In addition, a rice lateral rootless mutant *Lrt1* could be rescued by IBA but not IAA treatment (Chhun *et al.*, 2003). The mutated gene has yet to be described.

In contrast to lateral root development, adventitious root formation has significant practical implications because of the many plant species that are difficult to root. IBA is now used commercially worldwide to root many plant species (Hartmann *et al.*, 1990). However, *Arabidopsis* as a model to study adventitious rooting has so far been neglected. The aim of this study was 2-fold: (i) to analyse the process leading to adventitious roots on *Arabidopsis* stems and to find out which of the two auxins known to be present in *Arabidopsis* are involved in the process and to devise an experimental system which could be used to distinguish between callus and root formation and between IAA and IBA in the rooting process; (ii) to test this system for its use in the isolation of differentially expressed transcripts specifically involved in the rooting process. These transcripts could allow a more detailed analysis of adventitious rooting at the molecular level and help to identify candidate genes important for this process. The possible function of the transcripts isolated in this study for the rooting process will be briefly discussed. Furthermore, this system is also suitable for the analysis of available *Arabidopsis* mutants or chemical inducers or inhibitors of the rooting process.

It was shown that IAA and IBA were able to induce adventitious roots on cuttings of *Arabidopsis* stems if the segments were not removed during the treatment (Fig. 1), whereas removal of the segments from auxin-containing medium to MS medium only resulted in the production of calli with about the same efficiency for both hormones. Callus formation preceded adventitious rooting (Fig. 2). After shorter incubation times only IBA treatment resulted in the formation of roots (Fig. 3), indicating that IBA is an important factor for rooting. Several possibilities exist to explain the better performance of IBA versus IAA (summarized in Epstein and Ludwig-Müller, 1993): (i) higher stability, (ii) differences in metabolism, (iii) differences in transport, and (iv) IBA is a slow release source of IAA.

There is now a great deal of evidence that IBA occurs naturally in plants. The higher stability of IBA, in contrast to IAA, during rooting assays was reported by Nordström *et al.* (1991) which affected both degradation and metabolism. It was therefore suggested that IBA may be a very simple 'conjugate' of IAA and must be converted to IAA by β-oxidation to have an auxin effect. The conversion of IBA to IAA occurs in many plant species, such as *Malus pumila* (Alvarez *et al.*, 1989), *Pinus sylvestris* (Dunberg *et al.*, 1981), *Populus tremula* (Merckelbach *et al.*, 1991), *Pyrus communis* (Baraldi *et al.*, 1993), and *Vitis vinifera* and *Olea europaea* (Epstein and Lavee, 1984). However, in microcuttings of *Malus* it was found that IBA was converted to IAA only at very low levels (1%), but IBA itself induced more roots than IAA. This led the authors to suggest that either IBA itself is active or that it modulates the activity of IAA (van der Krieken *et al.*, 1992, 1993).

The transport hypothesis is supported by recent findings that IBA and IAA are differently transported in *Arabidopsis* (Rashotte *et al.*, 2003). These experiments are in agreement with this study's results using polar auxin transport inhibitors.

Several lines of evidence are now emerging which suggest that part of the effects of IBA are the direct action of the auxin itself (Ludwig-Müller, 2000; Poupart and Waddell, 2000), although other functions may be modulated by the conversion of IBA to IAA via β-oxidation (Zolman *et al.*, 2000; Bartel *et al.*, 2001). For example, drought and osmotic stress induced the synthesis of IBA and, consequently, the endogenous content of IBA was increased, whereas IAA was less affected (Ludwig-Müller *et al.*, 1995). In addition, IBA but not IAA was induced after the inoculation of maize roots with an arbuscular mycorrhizal fungus (Ludwig-Müller *et al.*, 1997; Kaldorf and Ludwig-Müller, 2000). In this paper a system was established for the induction of adventitious roots on sterile-grown stem sections of *Arabidopsis thaliana* where IBA induced adventitious roots under conditions where IAA was ineffective (Fig. 3). There was a desire to dissect the rooting process and therefore different time and concentration schemes were used for the optimization of adventitious root formation (Fig. 4), which allowed callus and subsequent root formation to be distinguished (Fig. 5).

The second goal of this research was the identification of differentially expressed transcripts during the rooting process. For this, the differential induction of callus and root on *Arabidopsis* stem segments were used and those treatments were compared with the controls (Fig. 6). Only those transcripts which showed up under treatment C (Fig. 6) were analysed further.

Initial studies on the hydrolytic enzymes found during root formation after IBA treatment in cuttings of mung bean revealed the induction of endo-β-1,4-glucanase (Shoseyov *et al.*, 1989), whereas the activities of β-1,3-glucanase and α-amylase were not affected. It was shown by *in situ* hybridization that the genes for endo-β-1,4-glucanase were expressed in the area of adventitious root primordia formation and in the cortex, where maceration of the cell walls was in progress in order to enable root emergence through the hypocotyl. To detect the induction of genes during adventitious root formation in loblolly pine (*Pinus taeda*) after treatment with IBA, a non-targeted approach via differential display reverse transcription-polymerase chain reaction was carried out (Hutchison *et al.*, 1999). One of the clones isolated by this method showed strong similarity to the α-expansin gene family of angiosperms and the differential gene expression after IBA treatment was confirmed by RNA blot analysis. Expansins are thought to be responsible for acid-induced cell wall loosening and are expressed in rapidly growing tissues (Cosgrove and Li, 1993; McQueen-Mason, 1995). They were reported to be induced in loblolly pine in non-growing regions of the stem prior to the resumption of cell division leading to the appearance of adventitious roots (Hutchison *et al.*, 1999).

One fragment differentially expressed during the adventitious rooting process in *Arabidopsis* (Fig. 6B) was identified as a regulatory subunit B of protein phosphatase 2A. In plants, type 2A serine/threonine protein phosphatases (PP2As) are critical in controlling the phosphorylation state of proteins involved in such diverse processes as metabolism, cell–cell communication, response to hormone, and auxin transport (Smith and Walker, 1996). The specificity, activity and subcellular targeting of PP2A is modulated by its association with the A and B subunits (Kamibayashi *et al.*, 1994). In *Arabidopsis*, three families of B-type regulatory subunits were identified, each consisting of more than one member (Corum *et al.*, 1996; LaTorre *et al.*, 1997; Rundle *et al.*, 1995; Sato *et al.*, 1997). Expression analysis indicated that, in plants, every B subunit shows a widespread, but fine-tuned, expression pattern in different organs (Thakore *et al.*, 1999). The function of PP2A during polar auxin transport has recently received more attention (Muday and DeLong, 2001, and references therein). One *Arabidopsis* mutant that provided insight into the regulation of auxin transport is called *roots curl in NPA1 (rcn1)*. This mutant was isolated using an assay for alterations in differential root elongation in the presence of the auxin transport inhibitor NPA aimed at isolating genes encoding proteins involved in auxin transport or its regulation. The *RCN1* gene encodes a regulatory A subunit of PP2A and the *rcn1* mutant exhibits reduced PP2A activity in extracts (Deruère *et al.*, 1999). The phenotypic alterations in this mutant are consistent with reductions in PP2A activity because treatment of wild-type plants with the phosphatase inhibitor cantharidin produces a phenocopy of *rcn1*. The *RCN1* gene is expressed in the seedling root tip, the site of basipetal transport, in lateral root primordia, and in the pericycle and stele, the likely site of acropetal transport (Muday and DeLong, 2001). It can be hypothesized that other PP2A subunits are co-ordinately expressed and that polar auxin transport also plays a role in adventitious root formation in *Arabidopsis*. This assumption is supported by the observation here that the auxin transport inhibitor TIBA inhibited adventitious root formation. Deduced from the findings summarized above a role can be proposed for PP2A in the regulation of auxin transport during adventitious rooting by altering the phosphorylation status of proteins involved in these processes thus most likely acting upstream of auxin transport. Auxin transport itself might be important for adventitious rooting by increasing local auxin concentrations.

A second fragment was identified as derived from an early-responsive dehydration stress ERD3 with otherwise unknown function (http://www.tigr.org/tdb/e2k1/ath1/). The sequence contains also a methyltransferase motif. Protection against dehydration may result in an increase

of lateral or adventitious root formation. It was shown that IBA synthesis was increased under drought stress in maize (Ludwig-Müller *et al.*, 1995) and the root system under these conditions was shorter, but with considerably more lateral roots. Drought rhizogenesis is an adaptive strategy that occurs during progressive drought stress and is characterized in *Arabidopsis* and other Brassicaceae and related families by the formation of short tuberized hairless roots (Vartanian *et al.*, 1994). These roots are capable of withstanding a prolonged drought period and give rise to a new functional root system upon rehydration. IBA might play a role during this process by inducing new roots. This protein might therefore play a more general role in IBA-induced root formation. As long as the function of ERD3 is unclear, this has to remain a hypothesis.

The Histidine Triad (HIT) motif identified in the third gene product, His-phi-His-phi-His-phi-phi (phi, a hydrophobic amino acid), was identified as being highly conserved in a variety of organisms (Seraphin, 1992). The crystal structure of rabbit Hint (histidine triad nucleotide-binding protein), purified as an adenosine and AMP-binding protein, showed that proteins in the HIT superfamily are conserved as nucleotide-binding proteins (Brenner *et al.*, 1997). Hint homologues hydrolyse adenosine 5′ monophosphoramide substrates and function as positive regulators of Cdk7/Kin28 *in vivo* (Bieganowski *et al.*, 2002), and Fhit (fragile histidine family) homologues related to the HIT family are diadenosine polyphosphate hydrolases (Barnes *et al.*, 1996). Therefore, the role of this protein during adventitious root formation might be in the regulation of the cell cycle or in signal transduction pathways.

In conclusion, it has been shown that it was possible to dissect the adventitious root formation process in *Arabidopsis* in such a way as to distinguish between the action of the two auxins IAA and IBA and to establish conditions where one hormone treatment arrests the process at the callus formation stage, whereas a second hormone treatment induces the formation of roots from these calli. In addition, it has been shown that the experiments presented here are a promising method to identify IBA-induced transcripts during adventitious root formation in the model plant *Arabidopsis thaliana*. To study the process of adventitious root formation further, several experiments can be envisioned: (i) the isolation of additional differentially expressed fragments from this screen, or using the now available microarrays to increase the number of cDNAs; (ii) using this screening method to identify *Arabidopsis* mutants impaired in adventitious root formation; and (iii) using known *Arabidopsis* mutants to investigate their response to IBA in this system. The gene sequences identified can then be used to probe the adventitious rooting pathway in horticulturally important species that are difficult to root.

Acknowledgements

This research was funded by Grant No. IBN 93-20210 from the National Science Foundation. JLM received a travel grant from the Dr Senckenbergische Stiftung, Frankfurt, Germany.

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Plant Cell, Tissue and Organ Culture 79: 239–247, 2004.

Approaches on vegetative propagation of difficult-to-root *Salix caprea*

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Key words: cutting propagation, growth regulator, *Melampsora*, micropropagation, sallow, tissue culture, willow rust

Abstract

In the framework of a willow rust research project, it was necessary to include vegetatively propagated plant material of selected sallow trees (*Salix caprea* L.) into biotests for identification of pathotypes. Since it was not possible to root sufficient clonal plants by conventional cutting propagation, the applicability of tissue culture methods was tested. From 10 selected donor trees of *Salix caprea* newly sprouted shoots were harvested and transferred to nutrient media after surface disinfection. The cultures were grown at 20–22 °C, illuminated with warm-white fluorescent tubes. The majority of shoot tips and nodal segments died during the first month, but only with nursery-grown plants this was caused by bacteria contamination. Two clones could be established easily on hormone-free medium. Five clones could be initiated only after repeated subcultures on various media variants. Three clones failed completely. Different basic media compositions were tested and Woody Plant Medium, supplemented with 0.1% activated charcoal, proved to be best for most of the sallow clones. Well developed rooted plantlets were used *in vitro* for microcutting propagation. The resulting plants were transferred to soil and could be included in the rust screening program after acclimatising. The applicability of micropropagation for selected *Salix caprea* donor trees was strongly depending on the genotype. But the comparison of results from microcuttings with conventional cutting propagation showed that these methods were successful for different clones each.

Abbreviations: BA – 6-benzyl-aminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; DKW – medium according to Driver and Kuniyuki (1984); GD – medium according to Gresshoff and Doy (1972); IBA – indole-3-butyric acid; MCM – medium for conifer micropropagation (Bornman, 1983); SH – medium according to Schenk and Hildebrandt (1972); WPM – Woody Plant Medium (Lloyd and McCown, 1980)

Introduction

Willows (*Salicaceae*) represent a genus with a broad spectrum of tree and shrub species mainly distributed over the northern continents. Depending on their properties and growth characteristics they are important for landscape development and establishment of ecological stable ecosystems, but also as planting stock for short-rotation coppice (SRC) plantations and as raw material for pharmaceutical applications of the wood components.

One of the most serious diseases on *Salix* are rust fungi of the genus *Melampsora*. These rusts are destructive plant pathogens and have been responsible for economic losses of SRC plantations. Infestation on a large scale with these fungi can cause premature defoliation and decreasing winter hardening. The use of molecular genetic techniques offers the possibility to understand the spectrum of the involved rust fungi more exactly (Pei et al., 1997; Liesebach et al., 2001). This is a prerequisite for resolution of these phylogenetic and pathological studies. Additionally pathoge-

nicity tests using leaf disc techniques (Pei et al., 1999) have to be realised on a range of willow clones from different species. Therefore, the multiplication of selected willow clones was necessary as the basis for a rust screening program. Although most of the *Salix* species are capable of natural vegetative regeneration, few members of the genus, e.g. *Salix caprea* and its hybrids, especially male plants, are difficult to root by conventional cutting propagation techniques (Chmelař, 1967; Neumann, 1981; Schiechtl, 1992; Mac Carthaigh and Spethmann, 2000).

Compared with other broad-leaved tree species investigations on tissue culture of the genus *Salix* were published less frequently. Bergman et al. (1985) tested the influence of the plant growth regulator BA on micropropagation potential of five different willow clones, including one of *S. caprea*. Neuner and Beiderbeck (1993) analysed nine clones of *S. caprea* for their tissue culture ability. Both publications emphasised the difficulties in cutting resp. microcutting propagation of sallow and the strong influence of genotypes on rooting success in this species. On the other hand, several authors have reported about successful application of tissue culture methods for some other willow species like *Salix viminalis*, *S. alba* and *S. fragilis* (Chalupa, 1983; Read et al., 1989; Agrawal and Gebhardt, 1994).

In the present paper, besides propagation via wood cuttings the suitability of micropropagation methods was tested for *Salix caprea* clones, aimed at the preparation of sufficient test material for a rust screening program.

Material and methods

Plant material

Two *Salix caprea* clones, both being male flowering hybrids, grown in the willow collection of the Forest Botanical Garden Eberswalde were chosen for propagation by softwood cuttings and micropropagation. These two donor clones were not suitable to get hardwood cuttings, therefore eight *S. caprea* plants from two natural stands in Schleswig-Holstein were used to obtain hardwood cuttings and explants for tissue culture initiation (Table 1).

Cutting propagation

The two hybrids (K1 and K72) from Eberswalde were propagated by softwood cuttings. In the mid of August 2001 the youngest twigs were collected and kept in wet cloth. The next day about 20 cuttings per clone of 15 cm length were prepared. The basal end was dipped into rooting paste supplemented with 9.85×10^{-3} M IBA and the leaf-area was halved to reduce the activity of photosynthesis and evaporation. The softwood cuttings were sticked into a sand:soil mixture (2:1) (13 × 13 cm pots) and kept under plastic foil cover

Table 1. Clones used for vegetative propagation

Clone	Species	Collection site
K1	*S. caprea* L. × *S. viminalis* L. (= *S.* × *smithiana*)	Forest Botanical Garden Eberswalde/Brandenburg (52° 49′ N, 13° 47′ E, 35 m asl)
K72	*S. caprea* hybrid	
S1	*S. caprea*	Natural occurrence on an afforestation site Schädtbek/ Schleswig-Holstein (54° 18′ N, 10° 16′ E, 40 m asl)
S2	*S. caprea*	
S3	*S. caprea*	
S4	*S. caprea*	
V1	*S. caprea*	Natural occurrence on an afforestation site Vollstedt/ Schleswig-Holstein (54° 15′ N, 9° 49′ E, 14 m asl)
V2	*S. caprea*	
V3	*S. caprea*	
V4	*S. caprea*	

with 100% air humidity in the greenhouse for 4 weeks. Temperature ranged between 18 and 25 °C on average. Two months later the rooting success was evaluated.

Hardwood cuttings of the eight clones from Schädtbek and Vollstedt, four clones each, were collected at the end of January 2002. The cuttings were watered for about 24 h, the basal end of the cuttings was dipped into rooting paste as described above. Forty cuttings of each donor tree were sticked into containers (type Rootrainer Jumbo, RONAASH Ltd. Scotland, 1 l volume) with a sand:soil mixture (2:1) and regularly irrigated. About 1 and 3 months later the number of flushed plants was counted. At the second date the plants were taken out of soil and checked for rooting success. The success of rooting was analysed statistically using contingency tables, χ^2 test and Fisher's exact tests to compare pairs of clones. Also the 'relative risks' (odds ratio) were estimated and confidence limits for the odds ratio were computed (PROC FREQ procedure of the SAS program, SAS Institute Inc., 1989).

Micropropagation

Tissue culture initiation

In October shoot tips and nodal segments, 2–2.5 cm long, were taken from potted cuttings of the two willow clones K1 and K72 in the greenhouse. The biggest leaves were reduced in size to one-third. The explants were disinfected for 8–10 min in 0.25% mercury chloride with a few drops of Tween80 followed by rinsing them three times in sterile distilled water.

Willow twigs, harvested from eight adult donor trees on the locations Schädtbek and Vollstedt at the end of January 2002, were put into vessels filled with tap water in the greenhouse. After spraying with 0.2% Euparen (fungicide by Bayer, 50% dichlorfluanide, w/v) the twigs were covered with black plastic bags to force flushing. Four weeks later 2–2.5 cm long shoot tips and nodal segments were cut off and after reduction of the biggest leaves washed in 0.2% Euparen for 2 min. After drying with filter paper, the explants were disinfected for 9 min in 0.25% mercury chloride with a few drops of Tween80. Rinsing in sterile distilled water was carried out three times. The basal end of the explants was cut again before placing them on nutrient medium in culture tubes.

The first flushing shoots from potted cuttings in the greenhouse were used as explant source at the end of April. Surface disinfection was done like described above, but mercury chloride was applied for 8 min. In August shoots and nodal segments were taken from container-grown cuttings in the nursery, applying an extended disinfection period of 20 min with mercury chloride.

Five different nutrient media were used for the tissue culture establishment: MCM according to Bornman (1983), WPM by Lloyd and McCown (1980), DKW following Driver and Kuniyuki (1984), GD by Gresshoff and Doy (1972) and SH according to Schenk and Hildebrandt (1972). The media were prepared without any plant growth regulator, but 0.1% activated charcoal (Darco) and 2% sucrose were added and 10 g l^{-1} of SERVA agar (gel strength ~800) was used as gelling agent.

Because only a limited amount of plant material was at disposal, the number of replications in all *in vitro* initiation experiments was restricted, ranging between 5 and 15 per media variant and clone. Therefore a statistical analysis was not appropriate. The cultures were kept at 20–22 °C with a light intensity of 1600–1700 lux (27.5–28 µmol m^{-2} s^{-1}) supplied by warm-white fluorescent tubes during a 16-h photoperiod.

Shoot multiplication

Multiplication of shoots was achieved by cutting rooted shoots after elongation and transfer of the shoot tips to fresh nutrient medium. Shoot tips of the not spontaneously rooting clones were transferred to half concentrated WPM supplemented with 2.46×10^{-4} M IBA for one week and then put on the phytohormone-free medium again.

Comparison of basic media

The basic nutrient media MCM, WPM, DKW, GD and SH, prepared like described above, were examined for their suitability for growth of willow micro-shoots. For this experiment tissue cultures of the two willow clones K1 and K72 established during former approaches were used. Shoot tips, 1.5–2 cm long, were put on the five media variants with 13 replications per each medium. The same conditions for cultivation like during clone initiation were used. The shoot length was measured at the beginning of the experiment and 4 weeks later. Additionally, the number of rooted shoots was

counted. Statistical analysis was carried out using the Tukey–Kramer test with $\alpha = 0.05$ (PROC GLM procedure of the SAS program, SAS Institute Inc., 1989).

Acclimatisation

Well rooted, vigorous plantlets were transferred to a standard soil:sand mixture (3:1) and kept under high air humidity (80–85%) maintained by mist irrigation under plastic foil cover. About 3 weeks later, plantlets were acclimatised by successive reduction of air humidity. After hardening, the micropropagated plants were grown in the nursery.

Results

Cutting propagation

The number of rooted plants produced by softwood cuttings was poor. Less then 10% of the clones K1 and K72 were rooted. This number was too low and the approach was finished.

The hardwood cuttings collected from *Salix caprea* growing at the Schädtbek and Vollstedt stands flushed rapidly. After 4 weeks in the greenhouse, there were three clones with all 40 cuttings having already flushed. The clone with the highest number of unflushed plants (13 of 40 cuttings, clone S2) had male catkins. From the other clones between 1 and 7 cuttings had not flushed. At the second date of evaluation, 2 months later, this sight had changed again (Table 2). In the greenhouse, plants of three clones flowered, therefore in this case the sex was known.

The statistical analysis showed significant differences in rooting success between the clones ($\chi^2 = 44.73$). The results of comparisons in pairs of rooting success, which are significant ($\alpha = 0.05$), are listed in Table 3. The odds ratio provides an estimate of the relative risk when an event is rare. For example, this estimate indicates that the odds of rooting success is 0.0952 times lower for clone V1 compared with V4. However, the wide confidence limits, which are combined with higher odds ratios, indicate that these estimates have low precision. Between flushing and rooting is no correlation ($r_s = -0.359$).

Micropropagation

Tissue culture initiation

The success of *Salix caprea* clone establishment *in vitro* was rather poor and strongly depending on the genotype. It was possible to cultivate the two clones K1 and K72 *in vitro* on phytohormone-free nutrient media, but finally after a preceded empirical search for suitable media variants, including different growth regulator combinations.

Out of the eight donor trees originating from the locations Schädtbek and Vollstedt, only three could be established *in vitro* during the approaches in spring. For two other clones (S1, V1), longlasting attempts were necessary to maintain the plant material after culture initiation in August. In Table 4 the different reaction of the clones during the three initiation experiments is shown.

The reason for this failure was an unexplainable dying of explants in the majority of clones (Figure 1). The clones S3 and V2 were established successfully already after the first experiment, but clone V4 could be finally maintained *in vitro* after repeated attempts with explants from potted cut-

Table 2. Development of the hardwood cuttings from two sites in Schleswig-Holstein in the greenhouse (starting with 40 replications in each clone)

Clone (flower)	Flushed 4th March	Flushed 2nd May	Rooted 2nd May
S1 (has not flowered)	39	39	23
S2 (male)	27	38	8
S3 (female)	34	35	10
S4 (has not flowered)	35	33	6
V1 (has not flowered)	40	36	5
V2 (has not flowered)	33	30	19
V3 (has not flowered)	40	31	14
V4 (female)	40	30	24

Table 3. Comparisons in pairs of rooting success

Clone (poor rooting success)	Clone (good rooting success)	Fisher's exact test (p-value)	Odds ratio	95% confidence limits
V1	V4	0.00001	0.0952	0.0307–0.2950
S4	V4	0.00003	0.1176	0.0402–0.3443
S2	V4	0.0002	0.1667	0.0613–0.4531
S3	V4	0.0012	0.2222	0.0835–0.5776
V3	V4	0.0150	0.3590	0.1450–0.8890
V1	S1	0.00002	0.1056	0.0342–0.3260
S4	S1	0.00007	0.1304	0.0447–0.3805
S2	S1	0.0005	0.1848	0.0682–0.5006
S3	S1	0.0024	0.2464	0.0951–0.6380
V3	S1	0.0239	0.3980	0.1614–0.9817
V1	V2	0.0005	0.1579	0.0513–0.4858
S4	V2	0.0014	0.1950	0.0671–0.5669
S2	V2	0.0066	0.2763	0.1024–0.7456
S3	V2	0.0213	0.3684	0.1429–0.9500
V1	V3	0.0132	0.2653	0.0848–0.8298
S4	V3	0.0252	0.3277	0.1108–0.9691

Table 4. Results of clone establishment *in vitro* of *Salix caprea* after 1 year

Clone label	Source of explants			
	Flushing twigs in the greenhouse	Potted cuttings in the greenhouse		Potted cuttings in the nursery
	March	April	October	August
K1			X	
K72			X	
S1	U	U		X
S2	U	U		C
S3	X			
S4	U	U		
V1	U	U		X
V2	X			
V3	U	U		C
V4	U	X		

X – successful established, U – unexplainable dying, C – severely contaminated, empty fields – not tested.

tings in the greenhouse. Media preferences were detectable during initiation phase for S3 and V2. Thus V2 preferred the WPM medium, whereas S3 survived best on WPM and MCM medium (see Figure 1).

The highest contamination rate occurred when nursery plants were used as explant source, compared with very low contamination rates being recorded when greenhouse plants were taken.

Shoot multiplication

During *in vitro* establishment spontaneous rooting of shoot tips occurred in the clones K1 and K72, but only in two of the eight clones from Schädtbek (S3) and Vollstedt (V2). The spontaneous rooting success ranged between 80 and 100% on phytohormone-free medium MCM. Clone V4 did not root spontaneously, but with a 1-week induction period on IBA containing medium the rooting

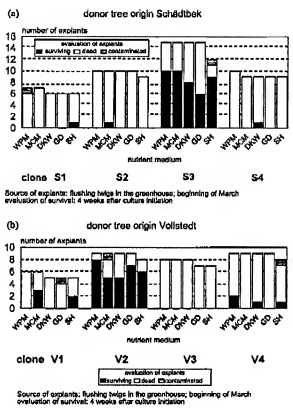

Figure 1. Evaluation of the *in vitro* establishment of *Salix caprea*.

results could be improved and ranged between 75 and 100%.

Multiplication of shoots was achieved by cutting the elongated, rooted shoots and transferring the shoot tips to fresh nutrient medium. The remaining stock plantlets were used repeatedly as donor plant for microcuttings during three subsequent subcultures (Figure 2). After three subcultures these mother-plantlets had to be substituted by newly rooted shoots for further supply of microcuttings.

Comparison of basic media

For the clone K1 the highest shoot elongation after 4 weeks was achieved with 17.4 mm on the WPM based nutrient medium (Figure 3), but the elongation difference was significant between WPM and DKW only. Clone K72 showed with 35.5 mm the significantly best shoot increment on WPM medium. Especially the medium DKW was less suitable because the shoots turned pale-green or yellow.

Acclimatisation

Shoots of the clones K1, K72, S3 and V2, which showed a good spontaneous rooting percentage and developed vigorous plantlets, were successfully transferred to the soil. After hardening in the greenhouse they were grown outdoors in the nursery (Figure 4).

Discussion

During the experiments presented here, only in two of the clones female catkins were detected. One of these clones (V4) showed better rooting success. Mac Carthaigh and Spethmann (2000) reported that propagation by cuttings is, especially for a few female sallow genotypes with green and

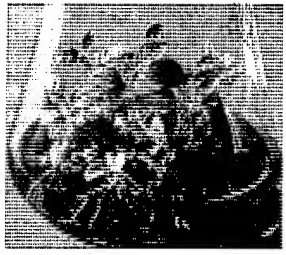

Figure 2. Sallow microcuttings (clone V4) on phytohormone-free medium, rooted after short-term-induction on medium containing 2.46×10^{-4} M IBA.

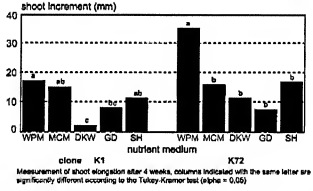

Figure 3. Influence of basic nutrient media on shoot elongation of *Salix caprea* L. *in vitro*.

not felty twigs, easily possible. Female clones of *S. caprea* are of low economic relevance (Mac Carthaigh and Spethmann, 2000). Sallows produce many seeds, therefore vegetative propagation is out of general interests.

Former experiments to transfer the two adult trees K1 and K72 into tissue culture resulted in preliminary hints that these *Salix caprea* clones are not susceptible for any of the phytohormone-containing nutrient media that were used efficiently for most of the broad-leaved tree species like birch (Ewald et al., 2000), poplar, black locust (Naujoks et al., 2000) or oak (Ewald and Naujoks, 1998). The only way to keep the cultures *in vitro* was a cultivation on hormone-free medium, preferably containing activated charcoal. In the experiments presented here, four out of 10 clones responded with rapid shoot and root development. The most suitable basic media were WPM and MCM, prepared with addition of 0.1% activated charcoal. Several authors were working on tissue culture of different willow species, but only two references dealing with sallow were found. Neuner and Beiderbeck (1993) analysed nine clones of *Salix caprea* for their tissue culture ability and reported that the addition of BA and kinetin had no positive effect on axillary or adventitious shoot production. They emphasised the unusual strong genotype-depending propagation and rooting behaviour of the species *Salix caprea in vitro*.

Figure 4. Sallow plantlets from micropropagation, four weeks after transfer to the soil (left) and after 1 year in the nursery (right).

Bergman et al. (1985) showed a stimulating effect of low concentrations of BA (5×10^{-7} M; 10^{-6} M) on the axillary shoot elongation of five clones of *Salix*, including one clone of *S. caprea*, but no adventitious bud formation could be reported. Gebhardt (1992) described browning of shoot tips from different willow species (e.g. *Salix viminalis, S. fragilis, S. petandra*) on cytokinin-containing medium and observed good shoot development and rooting of these clones on medium without phytohormones. These results are in contrast to Stoehr et al. (1989) who used WPM supplemented with BA and 2,4-D for callus induction with *Salix exigua*. Subsequent shoot regeneration was then achieved with BA alone. Agrawal and Gebhardt (1994) reported about a successful application of 0.2 mg l^{-1} BA for effective micropropagation of hybrid willows (*Salix fragilis* × *S. lispoclados*) from ovary culture and got a shoot multiplication rate of 5–8 until the third subculture.

For several broadleaved tree species thidiazurone was reported as an efficient nutrient media compound to substitute BA in micropropagation. This cytokinin-like acting growth regulator should be tested for sallow clones in future too, when shoots have been multiplied in a larger scale. The micropropagation method presented here can be used to facilitate plant production of selected *Salix caprea* clones. After successful establishment of microcutting mother-plantlets the system is more effective than rooting of hardwood cuttings since the propagation cycle can be continued over years and is independent from seasons.

Conclusion

It can be concluded that *Salix caprea* generally seems to be a species that is recalcitrant against vegetative propagation methods, but a few genotypes show a good response to cutting or microcutting propagation. Within the rust screening program micropropagated sallow plants could be already included on a small scale.

Acknowledgement

The research was funded by the EC and is part of the joint project 'Integrated, non-fungicidal control of *Melampsora* rusts in renewable energy willow plantations' (QLK5-1999-01585).

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AMENDMENT

Atty. Dkt. No. 081356-0210

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kazuko SHINOZAKI *et al.*

Title: **PRODUCTION OF PLANTS HAVING IMPROVED ROOTING EFFICIENCY AND VASE LIFE USING STRESS-RESISTANCE GENE**

Appl. No.: 10/798,579

Filing Date: 3/12/2004

Examiner: Vinod Kumar

Art Unit: 1638

Confirmation Number: 6471

AMENDMENT AND REPLY UNDER 37 CFR § 1.116

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The present communication is responsive to the final Office action dated March 1, 2010, concerning the captioned application. This response is timely filed by the first, extendible reply deadline of June 1, 2010.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this document.

Remarks/Arguments begin on page 4 of this document.

AMENDMENTS TO THE CLAIMS

This listing of claims replaces all prior versions of claims in the application.

1. (Currently Amended) A method of ~~producing~~ propagating a transformed plant, comprising:

(a) providing a plant material that is transformed with a heterologous DNA encoding DREB1A protein, wherein said DNA is under the control of a rd29A promoter;

(b) obtaining a scion from a mother plant produced from said plant material; and

(c) ~~producing~~ propagating a plant from said scion, such that said plant has a characteristic selected from (i) ~~improved propagation efficiency of scions, (ii)~~ improved propagation efficiency ~~and rooting efficiency~~ of scions, and ~~(iii)~~ (ii) improved propagation efficiency of scions and prolonged vase life of cut flowers, relative to a plant that is not transformed with said DNA, wherein each of (i) and (ii) is by way of improved rooting efficiency of scions for rooting.

2.-3. (Cancelled)

4. (Previously Presented) The method of producing a transformed plant of claim 1, wherein the DNA is selected from the group consisting of:

(a) a DNA consisting of the nucleotide sequence represented by SEQ ID NO: 1; and

(b) a DNA encoding a protein consisting of the amino acid sequence represented by SEQ ID NO: 2.

5.-13. (Cancelled)

14. (Previously Presented) The method of claim 1, wherein the DNA is transformed into the plant by using a vector selected from the group consisting of a virus, a Ti plasmid of Agrobacterium and an Ri plasmid of Agrobacterium.

15. (Previously Presented) The method of claim 1, wherein the DNA is transformed into the plant by electroporation, polyethylene glycol-mediated transformation, particle gun transformation, microinjection, silicon nitride whisker-mediated transformation, or silicon carbide whisker-mediated transformation.

REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and the following reasons.

I. Status of the Claims

Claims 2, 3, 5, 7, 8, and 10 were cancelled previously. Withdrawn claims 11-13 are cancelled in this response, pursuant to the examiner's request. Applicants reserve the right to file one or more continuing or divisional application to pursue the subject matter of any cancelled claims. Claim 1 has been amended for greater clarity.

Applicants acknowledge the finality of the outstanding Office Action. The claim revisions introduce no impermissible new matter and require no additional search, but they do place the application in condition for allowance or, at least, in better condition for appeal. Thus, applicants request entry of this amendment. Upon entry, claims 1, 4, 14, and 15 will be pending.

II. Rejection of Claims under 35 U.S.C. §103(a)

A. Kasuga and Byrne

Claims 1, 4, and 14 are rejected for alleged obviousness over Kasuga *et al.*, *Nature Biotechnology* 17: 287-91 (1999), in view of U.S. patent No. 5,584,140 to Byrne *et al.* Claim 15 is rejected over Kasuga in view of Byrne and Dalton *et al.*, *Plant Science* 132: 31-43 (1998). Applicants respectfully traverse each rejection.

(i) One skilled in the art would not have had any reason to combine the teachings of the cited references, thereby arriving at the claimed invention.

Kasuga describes a transgenic herbaceous plant produced by transforming *Arabidopsis* with DREB genes, thereby to impart drought, salt, and freezing tolerance. See the cited reference in the abstract and in the paragraph bridging pages 287 and 288.

Examiner Kumar has acknowledged that Kasuga fails to teach obtaining a scion from disclosed, transgenic *Arabidopsis* plant. See final action at page 5, first full paragraph. So saying, the examiner is understood to invoke Byrne for conventional usage of scions, obtained as cuttings from a mother plant, for grafting-based propagation. *Id.*, second full paragraph.

According to the examiner, one skilled in the art would have been motivated to propagate the transgenic *Arabidopsis* plant by Byrne's method to "eliminat[e] the expensive and time consuming steps of plant tissue culture and transformation." *Id.*, last paragraph. Yet, propagation of *Arabidopsis* material from scions is not mentioned in any cited reference.

Examiner Kumar seems not to intend a reliance in this regard on "Common Knowledge in the Art or 'Well Known' Prior Art," pursuant to MPEP § 2144.03. Accordingly, he must be understood to argue that the skilled artisan would have generalized Byrne's rooting method for vegetative plant propagation of hard-to-root plants, on "efficiency" grounds, to any and all plant types, including *Arabidopsis*.

This proposition not only is unsupported on the record, however, but also is erroneous as a matter of fact.

First, and more generally, propagation by scions or cuttings is not universally desirable, because some plant species or breeds develop bad rhizogenesis from scion propagation. Consequently, problems associated with the survival rate arise, due to decreased rooting and propagation efficiency.

Second, and more specifically, Byrne's propagation methodology would not have been readily applicable to Kasuga's transgenic *Arabidopsis* plant. Thus, as Byrne's abstract indicates, the prior-art method entails developing etiolated shoots on stock plants, removing those shoots, developing roots from the shoots, and then planting the rooted shoots. Tables 1-3 exemplify plants that are suitable for such manipulations, and all are woody plants, such as apple, beech, birch, and chestnut. In keeping with this orientation, Byrne further requires that the stock plant

be grown to a mean diameter of at least ¼ inch and a height of 3 feet (column 8, lines 2-5), and that cuttings not be done until the shoots are in transition from a "softwood" stage to "semi-hardwood" stage (column 10, lines 3-10).

These softwood/hardwood directions would have been meaningless with regard to herbaceous plants in general and particularly to *Arabidopsis*, which usually grows to maximum height of only 20 to 25 cm (for instance, see Figures 1 and 3 of Kasuga). It is not surprising, therefore, that Byrne fails even to hint at how his methodology might apply to a herbaceous plant. Indeed, the aforementioned teachings, if anything, would have directed the skilled artisan *away* from thinking that Byrne's propagation method for woody plants could applied to advantage with respect to Kasuga's transgenic *Arabidopsis* plant.

Given these defects in the evidentiary record, applicants are obliged to point out the decidedly *ad hoc* cast of the examiner's rationale. In fact, only impermissible hindsight could explain why one of ordinary skill would have transitioned from Kasuga's work with a model organism, *Arabidopsis thaliana*, to an *a priori* reasonable expectation of some desirable result achieved by propagating DREB-transformed plant material via scions, a technique associated primarily with tree and shrub husbandry.

To substantiate the rejection, therefore the examiner has made a legal error by breaking the claimed invention into elements, looking for each element in prior art, and then assembling the elements in accordance with a road map provided by Applicants' claimed invention. For this reason alone, the rejection should be withdrawn.

(ii) The combined teachings of the cited references fail to render the claimed invention obvious.

The examiner asserts that one skilled in the art would have expressed Kasuga's DREB gene in a plant to obtain the stress-resistant features, and that such plant also would have exhibited other characteristics, including improved rooting efficiency and prolonged vase life of flowers. See final action at page 6, first paragraph.

In fact, neither of the cited references suggests that introducing DREB gene would have any effect on rooting of scions or prolonging vase life of cut flowers. This is hardly surprising , since Kasuga's transgenic *Arabidopsis* plant was incapable of generating cut flowers.

The skilled artisan might well have understood that promotion of rooting might serve the ends of improved drought resistance, for example, but there was no basis the art for predicting that DREB expression could enhance rooting. As noted above, *Arabidopsis* also would not have been deemed a ready target for scion-based propagation. To the contrary, it was impossible before the present invention to associate (A) the fact that placing DREB1A-encoding DNA under the control of a rd29A promoter, as presently recited, would effect expression of stress-responsive proteins with (B) the promotion of rooting. Put another way, there was no reason for the skilled artisan to have looked to scion-based propagation of any transgenic plant, let alone one that expressed DREB1A-encoding DNA, in relation to solving a problem of lowered survival rate for rooting. Again, the examiner must rely on hindsight, *sub silencio*, to make these connections.

Dalton is cited for the alleged teaching of plant transformation methods prescribed by claim 15. Even take at face value, however, the examiner's reading of Dalton does not compensate for the above-discussed deficiencies in the primary and the secondary references. Accordingly, claim 15 is allowable as well over the cited art.

B. Shinozaki '742 and Byrne

Claims 1, 4 and 14 are rejected over U.S. Patent No. 6,495,742 to Shinozaki *et al.* in view of Byrne, *supra*. Claim 15 is rejected separately over Shinozaki '742 in view of Byrne and Dalton, discussed above. Applicants respectfully traverse each rejection.

Shinozaki's disclosure is similar to that of Kasuga. The examiner has advanced essentially the same rejection rationale, which is addressed in section A above. Accordingly, all arguments above are incorporated by reference.

Shinozaki ;742 additionally discloses that the host plant may be *Arabidopsis thaliana*, tobacco, rice and maize (see column 12, lines 4-5), but there is no suggestion that such plants might be propagated advantageously by scions. Each of the aforementioned hosts is a herbaceous plant.

As discussed above, the examiner has not yet established why the skilled artisan would have applied methodology suited to woody plants, per Byrne, for propagating Shinozaki's herbaceous transgenic plants. Moreover, even were the cited references so combinable, which they are not, one of ordinary skill in the art would have lacked basis for reasonable expectation of thereby obtaining transgenic plants characterized by improved rooting efficiency and prolonged vase life of cut flowers, given prior-art transgenic plants that were resistant to drought, salt, and freezing. Claim 15 is allowable for the same reasons, as Dalton does not remedy the deficiencies of Shinozaki and Byrne.

C. Shinozaki '528 and Byrne

Claims 1, 4, and 14 are rejected over U.S. Patent No. 6,670,528 to Shinozaki *et al.* in view of Byrne. Claim 15 stand rejected over Shinozaki in view of Byrne and Dalton. Applicants respectfully traverse each rejection.

Shinozaki '528 is cumulative of Shinozaki '742 and Kasuga, which are discussed in detail above. The secondary and tertiary references are the same, and the examiner has advanced essentially the same rationale to support this rejection. Accordingly, all arguments in sections A and B above are incorporated by reference.

In view of the foregoing, withdrawal of Section 103 rejections is warranted.

CONCLUSION

The present application is now in condition for allowance, and an early indication to this effect is respectfully requested. Examiner Kumar is invited to contact the undersigned directly, should he feel that any issue warrants further consideration.

Respectfully submitted,

Date June 1, 2010

By 

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